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14. ABSTRACT
The goal of this project is to design and test novel synthetic peptides that can be cleaved by the extracellular enzyme Prostate Specific Antigen (PSA) to yield a peptide fragment that can enter cells and directly induce apoptosis. The objective is to explore a new approach for developing molecularly targeted systemic agents for metastatic prostate cancer. The synthetic peptides were designed with domains that could be (i) cleaved by PSA, (ii) permeate the plasma membrane, and (iii) disrupt mitochondrial membranes to induce apoptosis. Cell lines on which the synthetic peptides will be tested (human prostate cancer cells, normal primary human prostate epithelial cells, and human endothelial cells) were obtained and optimal culture conditions were determined. Optimal assays for cytotoxicity or apoptosis for each cell line were determined. Testing of the peptides will be performed in the final six months of the project.

15. SUBJECT TERMS
PSA cell-permeable peptide apoptosis

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Introduction

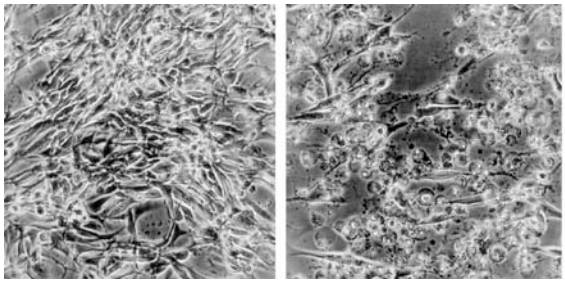
Prostate cancer is the most common cancer in men, accounting for 33% of all cancer diagnoses and 9% of all cancer deaths in men (1). New therapies are urgently needed for metastatic prostate cancer, which is frequently fatal. Prostate cancer cells secrete the enzyme Prostate Specific Antigen (PSA) which normally cleaves its substrate semenogelin. One approach that has been taken to develop new therapies for prostate cancer is the development of PSA-activated pro-drugs. These are agents that covalently link peptide sequences from semenogelin with a cytotoxic agent, and are designed to become cytotoxic only after cleavage by PSA. A second approach to new therapies is the development of cell-permeable pro-apoptotic peptides. These are small peptides (10 to 40 amino acids) that have been shown to enter cells and induce apoptosis by disrupting mitochondrial membranes thereby activating intrinsic apoptosis signaling pathways. The goal of this Exploration-Hypothesis grant is to investigate the feasibility of combining these two approaches by designing and testing a PSA-activated pro-apoptotic peptide. Ideally it would be a non-toxic synthetic peptide that is specifically cleaved by PSA to produce a cell-permeable fragment that induces apoptosis in prostate cancer cells and endothelial cells. The scope of this project is to culture cells in vitro (prostate cancer, normal prostate, and endothelial cells), determine the optimal methods for quantifying cell death in each cell type, and then test the novel synthetic peptides I designed to determine if they induce cell death when cleaved by PSA.

Body

A prerequisite for testing the synthetic peptides is growing the four cell types to be tested in culture, including: prostate cancer cell lines PC-3 and LNCaP, normal primary prostate epithelial cells, and human endothelial cells. Culture conditions for PC-3 and LNCaP prostate cancer cells are established, and these cells were grown without problem. Culturing normal primary prostate epithelial cells proved more challenging. The cells were obtained from Clonetics and initially were grown in the manufacturer's supplied medium. However, the growth characteristics in this medium were suboptimal, and cells stopped growing after only 1 to 2 passages. I explored a number of variables to determine how best to sustain growth, including different plating densities, smaller plates, avoiding the use of trypsin, or using coated plates. Ultimately I found that increasing the concentration of fetal bovine serum from 2% to 10%, removing cells from the plate with EDTA/PBS instead of trypsin, and splitting the cells no greater than 1:3 could support continued growth for up to 5 passages. Culturing human endothelial cells also required optimization of conditions. Human endothelial cells were also obtained from Clonetics and had a limited lifespan using the manufacturer's medium. After investigating a series of cell culture conditions I ultimately found that supplementing the medium with additional VEGF and additional fetal bovine serum roughly doubled the number of passages before reaching senescence.

Culturing cells should not have been particularly complicated because I have done cell culture for years. But in the course of working out optimal cell culture conditions I saw recurring episodes of contamination most likely by molds. Simple measures taken to solve the problem (e.g., new medium and serum, flaming Pasteur pipet tips) didn't eradicate the problem. Taking additional measures to ensure sterility (always thoroughly wiping down the hood and gloves, recleaning the cell culture incubators and water baths, restricting mentored high school students from using the hood, etc.) did not resolve the problem. Ultimately, I had the tissue culture hood repair service decontaminate the hood in February and replace the filters. The invoice for this work is attached in the appendix. This finally resolved the problem but working through the situation set back the project timeline. I had used this hood for six years without problems so I didn't immediately suspect it to be the cause.

Identifying optimal cell death or cytotoxicity assays: In order to test the synthetic peptides for their ability to induce cell death, I needed to identify a sensitive, reproducible, and ideally rapid assay for cell death or cytotoxicity for each cell type. Many different assays are described in the literature and there are at least 25 that are commercially available, but no single assay is best suited for all cell types. Because the peptides to be tested are expensive, I focused on cell death or cytotoxicity assays that are done in 96-well plates in a volume of 50 to 100 µL so as to minimize the amount of peptide that will be consumed in each experiment when they are tested. I began the process of evaluating cell death and cytotoxicity assays by determining in preliminary experiments that all four cell types (LNCaP and PC-3 prostate cancer cells, normal human primary epithelial prostate cells, and human endothelial cells) were almost completely killed by treatment for 24 hours with the chemotherapy agent camptothecin at 20 µM. Camptothecin is a topoisomerase inhibitor that activates the intrinsic apoptotic signaling pathways, as the synthetic peptides are designed to do. An example of camptothecin killing PC-3 cells is shown here:

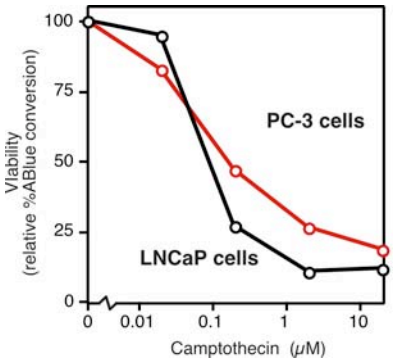


PC-3 cells were treated for 24 hours with camptothecin (20 μM, right panel) or left untreated (left panel). Camptothecin induced substantial cell death in all cell types.

Using camptothecin as a standard and reproducible method to kill the four cell types, I then evaluated a series of cell death or cytotoxicity assays that are performed in 96-well plates and read on a plate reader. The protocol involved plating each cell type at 10,000 cells/well in a 96-well plate and treating for 24 hours with graded concentrations of camptothecin (0 to 20 µM) to produce a range of cell death responses from no death to nearly complete cell death. Then

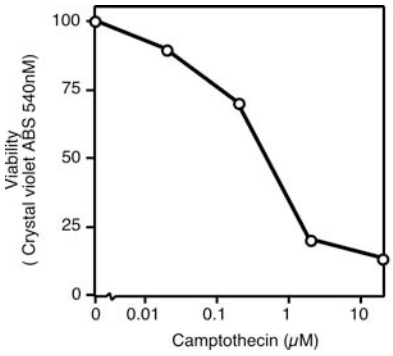
the cell death or cytotoxicity assays were performed to assess the sensitivity and reproducibility of the assay on that cell type.

As expected, no single assay worked best for all four cell types. The AlamarBlue/resazurin assay, a variation of the MTT assay, worked well for LNCaP cells and PC-3 cells, as shown below. Other assays, including LDH release, WST-1, crystal violet, or trypan blue were less optimal for a variety of reasons, including lower sensitivity, variable results, high background, cost, or were very labor intensive.



PC-3 and LNCaP prostate cancer cells were plated at 10,000 cells/well in 96-well plate and treated with indicated concentration of camptothecin. AlamarBlue assay was performed at 24 hours.

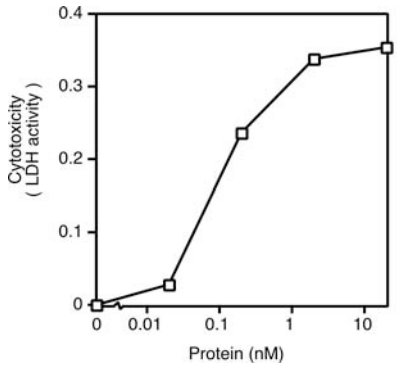
For normal primary prostate epithelial cells, the best performing assay was Crystal violet. Representative example shown here:



Normal primary prostate epithelial cells were plated at 10,000 cells/well in 96-well plate and treated with indicated concentration of camptothecin. Crystal violet assay was performed at 24 hours.

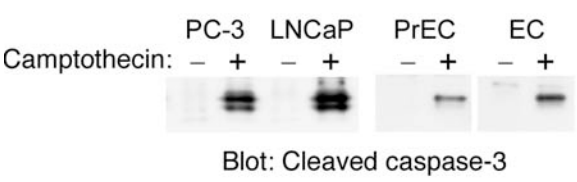
The other assays (AlamarBlue, LDH release, WST-1, or trypan blue) performed less well on these cells for some of the same reasons noted above.

For human endothelial cells, the most useful assay was LDH release. Unlike AlamarBlue or Crystal violet assays, which measure cell viability, the LDH release assay quantifies cell toxicity by measuring the release of intracellular LDH enzyme into the medium. Example shown here:



Human endothelial cells were plated at 10,000 cells/well in 96-well plate and treated with indicated concentration of camptothecin. LDH release assay was performed at 24 hours.

To establish that the synthetic peptides activate the intrinsic apoptosis pathway it will be necessary to demonstrate activation of caspase-3 or a similar downstream apoptosis effector molecule. To confirm that activated caspase-3 can be detected in the four cell types, I treated the cells with camptothecin and performed an immunoblot using an antibody that specifically recognizes cleaved and activated caspase-3. As shown, the antibody (Cell Signaling Technology) readily detected activated caspase-3.



PC-3, LNCaP, normal prostate epithelial cells, or endothelial cells were treated with campthecin (20μM) for 16 hrs or left untreated. Lysates were separated by SDS-PAGE and immunoblotted with antibody against cleaved/active caspase-3.

It was necessary to invest significant effort into identifying the best assays for cell death or cytotoxicity for each cell line because when testing the synthetic peptides the cell death or cytotoxicity response may be relatively weak. The progress made in the first twelve months was frustrating and slower than I had intended, especially the cell culture hood contamination problem. However I remain optimistic that many of the tasks outlined in the Statement of Work can be accomplished before the end of the grant.

Key research accomplishments to date

1. Determined optimal growth conditions for human prostate cancer cells, normal human prostate cells, and human endothelial cells.

2. Determined most sensitive and reproducible assay for cytotoxicity or apoptosis for prostate cancer cell lines, normal prostate cells, and endothelial cells.

Reportable outcomes

Funding applied for and granted:

Prostate Cancer Foundation
"Recombinant proteins that convert VEGF into a cell death factor as therapy for prostate cancer"
P.I. Timothy Quinn, M.D.
4/2007 – 4/2008

Conclusions

The chief importance of the completed work was to establish the cell culture methods and cell death or cytotoxicity assays that are required for testing the synthetic peptides.

References

1. Taichman RS, Loberg RD, Mehra R, and Pienta KJ. The evolving biology and treatment of prostate cancer. JCI 117:2351, 2007.

Appendices

Tissue culture hood decontamination invoice

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From: Liza Canlas [LCanlas@techsafety.com] Sent: Mon 2/12/2007 8:18 AM

To: Quinn, Timothy

Cc: Emil Forsblad

Subject: UCSF(USF070345A-01): Request PO#

Attachments:

View As Web Page

Morning Tim,

Here is the following information you will need to issue a Purchase Order number:

Service Order: USF070345A-01

Date of Service: 02/01, 02/02, & 02/05 2007

TOTAL AMOUNT DUE:$1,115.00

Task	Rate Type	Rate Factor	Quantity	Price Per Unit
Labor	Labor	Standard	7	$75.00
Decontaminate Biosafety Cabinet	Task Rate	Standard	1	$175.00

Freight: $25.00		
Part	**Quantity**	**Price Per Unit**
Motor	1	$345.00
Cap	1	$25.00

If you have any questions, feel free to contact me.

Thanks,

Liza-Marie Canlas

Administrative Assistant

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